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Gina N. Shishima

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Chada et al.

Serial No.: 10/017,472

Filed: December 7, 2001

For: METHODS OF TREATMENT INVOLVING HUMAN MDA-7

Group Art Unit: 1632

Examiner: Li, Qian J.

Atty. Dkt. No.: INGN:097US

DECLARATION OF SUNIL CHADA, Ph.D

I, Sunil Chada, declare:

1. I am the Director of Research and Development at Introgen Therapeutics. I have been working in the field of gene therapy and cancer biology for at least 15 years. My *curriculum vitae* is attached as Exhibit 1.

2. I am also one of the inventors named on the application identified above, which concerns the melanoma differentiation associated gene (mda-7) and its encoded protein, MDA-7.

3. The *mda-7* gene was first identified in human melanoma cell lines as a possible tumor suppressor. Jiang *et al.*, *Oncogene* 11:2477-86 (1995). Subsequent studies confirmed that elevated levels of MDA-7 suppressed cancer cell growth *in vitro* and selectively induced apoptosis in human breast cancer cells and inhibited tumorigenicity in nude

mice. Jiang *et al., Proc. Nat'l. Acad. Sci.* 93:9160-65 (1996); Su *et al., Proc. Nat'l Acad. Sci.* 95:14400-05 (1998).

4. I understand that the present application contains claims directed to methods of inhibiting angiogenesis involving administering a nucleic acid expressing the human MDA-7 polypeptide, which have been rejected as lacking enablement.

5. As described in this application, the first 48 amino acids of the full-length sequence may be cleaved to yield a secreted form of the protein. I have done scientific research on the tumor suppressor gene mda-7 and the MDA-7 protein, both the full-length and truncated versions.

6. In one study concerning the MDA-7 protein, human melanoma cell lines MeWo and WM35 were treated with increasing concentrations of an MDA-7 protein lacking the first 48 amino acids of the full-length sequence. The cell lines were analyzed in triplicate at 12, 24, 48, 72, and 96 hours after treatment using a trypan blue exclusion assay. This truncated MDA-7 protein induced cell killing in melanoma cells (Exhibit 2), but did not induce killing in lung cancer cells.

7. In another study, different forms of the MDA-7 protein were evaluated in PC3 human prostate cancer cells and H1299 human non-small cell lung carcinoma cells. The different forms (Exhibit 3) included: a full-length MDA-7, an MDA-7 protein lacking its own secretion signal (cytoplasmic version. lacking first 48 amino acids), an MDA-7 targeted to the nucleus (nuclear version), and an MDA-7 lacking its own secretion signal but containing a signal targeting it to the endoplasmic reticulum (ER version). Cells transfected with either the full-length or ER version of MDA-7 showed growth suppression (Exhibit 4). Furthermore, there were higher levels of apoptosis observed in

cells transfected with the full-length or ER versions, as compared to the cytoplasmic or nuclear versions of MDA-7.

8. Thus, as discussed in paragraph 6, the truncated version of MDA-7 does indeed induce apoptosis as set forth in the specification of this application. Furthermore, as discussed in paragraph 7, a truncated MDA-7 with a heterologous signal sequence suppresses growth and induces apoptosis.

9. Moreover, while the specification provides data regarding an Ad-mda7 construct to express MDA-7 in a eukaryotic cell, another study involved formulating a plasmid with an MDA-7 encoding nucleic acid in a liposome composition. The human mda-7 cDNA was placed under the control of the CMV promoter in a plasmid, which was formulated in a DOTAP:cholesterol complex. Nude mice were injected with human non-small cell lung carcinoma cells (A549 cell line) to produce tumors. Tumors were then treated intratumorally with the DOTAP:Chol-*mda-7* complex (50 μg/dose), resulting in the inhibition of tumor growth as compared to tumors in control animals. Similarly, tumors in nude mice from implantation of fibrosarcoma cells (UV223M cells) (syngeneic tumor model) were also inhibited by intratumoral administration of the DOTAP:Chol-*mda-7* complex. Moreover, when the tumor tissue from these animals were evaluated for CD31, they exhibited reduced levels of staining, which is indicative of reduced vascularization.

10. I hereby declare that all statements made of my own knowledge are true and all statements made on information are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code, and that such

willful false statements may jeopardize the validity of this application or any patent issued thereon.

01-29-04
Date

Sunil Chada, Ph.D.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
SUNIL CHADA	DIRECTOR OF RESEARCH AND DEVELOPMENT

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Kings College, University of London London, England	B.Sc. (Honors)	1982	Cell & Molecular Biology
University of California at Los Angeles Los Angeles, CA	M.Sc.	1985	Molecular Biology
University of Massachussetts Medical School Worcester, MA	Ph.D.	1988	Molecular Genetics

A.

B. **Positions and Honors.** List in chronological order previous positions, concluding with your present position. List any honors. Include present membership on any Federal Government public advisory committee.

PROFESSIONAL EXPERIENCE

1985-1988 Research Associate, Univ. of Massachusetts Medical School, Worcester MA
1988-1991 Research Scientist I, Dept. of Molecular Virology, Viagene Inc., San Diego CA
1991-1993 Research Scientist II, Dept. of Immunobiology, Viagene Inc., San Diego CA
1993-1995 Senior Scientist, Dept. of Immunobiology, Viagene Inc., San Diego CA
1995-1997 Staff Scientist, Chiron Technologies Inc., San Diego CA
1997-pres Director of Research and Development, Introgen Therapeutics, Houston TX
2002-pres Adjunct Faculty, Dept. of Bioimmunotherapy, Division of Cancer Medicine, MD Anderson Cancer Center

Committee Memberships

National Cancer Institute – SBIR/ STTR SRG Reviewer (standing member)
National Cancer Institute – Cancer Chemoprevention (Ad hoc member)
National Cancer Institute – RAID Committee member
Rice University – Advisory Board for NIH and NSF Biotechnology Training Programs
Alliance for Cancer Gene Therapy - Reviewer

c. **Selected peer-reviewed publications (from a total of 68).**

1) Chou C, Gatti RA, Fuller M, Concannon P, Wong A, **Chada S**, Davis R, and Salser W. "Structure and expression of ferritin genes in a human promyelocytic cell line that differentiates in vitro." *Molecular and Cellular Biology* 6:566-573 (1986).

2) Davis RC, Thomason AR, Fuller ML, Slovin JP, Chou CC, **Chada S**, Gatti RA, and Salser W. "mRNA species regulated during the differentiation of HL-60 cells to macrophages and neutrophils." *Developmental Biology* 119:164-174 (1987).

3) **Chada S**, Whitney C, and Newburger, PE. "Control of expression of the human glutathione peroxidase gene by selenium." In: *OxyRadicals in Molecular Biology and Pathology*, eds. Cerutti P, Fridovich I, and McCord J, UCLA Symposia on Molecular and Cellular Biology Vol. 82. Alan R. Liss, New York, pp.273-288 (1988).

4) Holland CA, **Chada S**, Wright J, Whitney C, Harigaya K, Greenberger J, Newburger PE. "Differentiation of Human Hematopoietic Cells Increases Expression of a Gene Transferred by a Retroviral Vector." *J. Leukocyte Biology* 46:221-229 (1989).

5) **Chada S**, Whitney C, and Newburger PE. "Post-transcriptional regulation of glutathione peroxidase gene expression by selenium in the HL-60 human myeloid cell line." *Blood* 74:2535-2541 (1989).

6) **Chada S** and Newburger PE. "Incorporation of selenocysteine at a UGA termination codon in the human glutathione peroxidase gene". In: *Glutathione Centennial; Molecular Perspectives and Clinical Implications*, eds. Taniguchi N, Higashi T, Sakamoto Y, and Meister A. Academic Press, Inc, San Diego pp:145-160 (1989).

7) **Chada S**, LeBeau M, and Newburger PE. "Isolation and chromosomal localization of the human glutathione peroxidase gene." *Genomics* 6:268-271 (1990).

8) Warner JF, Anderson CG, Laube L, Jolly D, Townsend K, **Chada S** and St. Louis D. "Induction of HIV-specific CTL and antibody responses in mice using retroviral vector transduced cells." *AIDS Research and Human Retroviruses* 7:645-655 (1991).

9) Jolly J, **Chada S**, Townsend K, DeJesus C, Chang S, Weinhold K, Anderson CG, Lynn A, Bodner M, Barber J, and Warner J. "CTL cross reactivity between HIV strains." *AIDS Research and Human Retroviruses* 8:1379-1381 (1992).

10) Warner JF, Anderson CG, Laube L, Jolly D, Irwin M and **Chada S**. "Immunotherapy using retroviral vectors" In "*Molecular Basis of Immune Responses*" eds. Nariuchi, H et al. Academic Press, Inc, San Diego. pp.155-165 (1993).

11) **Chada S**, DeJesus C, Townsend K, Lee W, Laube L, Jolly D, Chang S and Warner J. "Cross-reactive lysis of human targets infected with prototypic and clinical HIV-1 strains by murine anti HIV-1 IIIB env specific CTL." *Journal of Virology* 67:3409-3417 (1993)

12) Thor G, Sepulveda H, **Chada S** and Dutton RW. "A monoclonal antibody that distinguishes between a phosphorylated, beta-2 microglobulin associated and a non-phosphorylated, free heavy chain of MHC class I." *Journal of Immunology*.151:1-14 (1993)

13) Sajaddi N, Kamantigue E, Edwards W, Howard T, Jolly D and **Chada S**. "Recombinant retroviral vector delivered intramuscularly localizes to the site of injection in mice." *Human Gene Therapy* 5:693-699 (1994)

14) Irwin M, Laube L, Lee V, Austin M, **Chada S**, Anderson C-G, Townsend K, Jolly D and Warner J. "Direct injection of a recombinant retroviral vector induces HIV specific immune responses in mice and nonhuman primates". *Journal of Virology* 68:5036-5044 (1994)

15) Shen Q, **Chada S**, Whitney C and Newburger PEN. "Regulation of the human cellular glutathione peroxidase gene during monomyelocytic differentiation." *Blood* 84:3902-3908 (1994)

16) Newburger PEN, Malawista S, Dinauer M, Gelbart T, Woodman R, **Chada S**, Shen Q, von Blaricam G, Quie P and Curnutte J. "Chronic Granulomatous Disease and glutathione peroxidase deficiency, revisited" *Blood* 84:3861-3869 (1994)

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18) T. W. Dubensky, Driver DA, Polo JM, Belli BA, **Chada S**, Brumm D, Banks TA, Mento SJ, Jolly DJ and Chang SM. "Sindbis virus DNA-based expression vectors: utility for *in vitro* and *in vivo* gene transfer." *Journal of Virology* 70: 508-519 (1996)

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21) Kline RM and **Chada S.** "Killing The Last Cell: Using The Immune System To Fight Cancer." *MIT Technology Review.* June:48-55 (1997)

22) Nguyen KHY, Boyle DL, McCormack JE, **Chada S** and Firestein G. "Direct synovial gene transfer with retroviral vectors in rat adjuvant arthritis". *Journal of Rheumatology* 25: 1118-1125 (1998)

23) Ishida T, **Chada S**, Stipanov M, Nadaf S, Ciernik FI, Gabrilovich DI and Carbone DP. "Dendritic cells transduced with wild type p53 gene elicit potent anti-tumor immune responses". *Clinical and Experimental Immunology*. 117(2):244-51 (1999)

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25) Russell JS, Lang FF, Huet T, Janicot M, **Chada S**, Wilson DR and Tofilon PJ. "Radiosensitization of human tumor cell lines induced by the adenovirus-mediated expression of an anti-ras single chain antibody fragment". *Cancer Research*. 59(20):5239-44 (1999)

26) Saeki T, Mhashilkar AM, Roth JA,, Branch C, **Chada S** and Ramesh R. "Tumor-suppressive effects by adenovirus-mediated *mda-7* gene transfer in non-small cell lung cancer cell *in vitro*". *Gene Therapy* 7: 2051-2057 (2000)

27) Mhashilkar AM, Schrock RD, Hindi M, Liao J, Sieger K, Kourouma F, Zou-Yang H, Onishi E, Takh O, Vedvick T, Fanger G, Stewart L, Watson GJ, Snary D, Fisher PB, Saeki T, Roth JA, Ramesh R and **Chada S.** "Melanoma-Differentiation Associated Gene (mda-7): A Novel Anti-Tumor Gene for Cancer Gene Therapy". *Molecular Medicine,* 7(4): 271-282 (2001). Cover article.

28) Nikitina EY, Clark JI, Benyen J, **Chada S**, Virmani AK, Carbone DP and Gabrilovich DI. "Generation of Anti-tumor Cytotoxic T Lymphocytes from Peripheral Blood of Cancer Patients using Dendritic cells transduced with Wild Type p53 Gene". *Clinical Cancer Research*, 7:127-135 (2001).

29) Saeki T, Mhashilkar AM, Swanson X, Zou-Yang H, Zumstein L, Sieger K, Branch CD, Roth JA, **Chada S** and Ramesh R. "Inhibition of human lung cancer growth following adenovirus-mediated *mda-7* gene expression *in vivo*". *Oncogene* 21(29): 4558-66 (2002)

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40) Pataer A, **Chada S**, Hunt KK, Roth JA and Swisher SG. "Adenoviral MDA-7 induces apoptosis in lung cancer cells through Mitochondrial Permeability Transition independent Cytochrome C Release". *Journal of Thoracic and Cardiovascular Surgery* 126(6):1328-1325 (2003)

41) Sasaki JI, Ramesh R, **Chada S**, Gomyo Y, Roth JA, and Mukhopadhyay T. "An Anthelminthic Drug to Curb Human Cancer: A Novel Microtubule Inhibitor as a Potential Inducer of Apoptosis in Non-Small Cell Lung Cancer Cells but Not in Normal Cells *In Vitro* and *In Vivo*". *Molecular Cancer Therapeutics*. 1: 1201-1209 (2002)

42) Ekmekcioglu S, Ellerhorst JA, Mumm JB, Zheng M, Broemeling L, Prieto VG, Stewart AL, Mhashilkar AM, **Chada S** and Grimm EA. "Negative Association of Melanoma Differentiation Associated Gene (mda-7) and inducible Nitric Oxide Synthase (iNOS) in Human Melanoma: MDA-7 Regulates iNOS Expression in Melanoma Cells." *Molecular Cancer Therapeutics*. 2(1):9-17 (2003)

43) Ramesh R, Mhashilkar A, Tanaka F, Saito Y, Sieger K, Mumm JB, Stewart A, Branch CD, Boquio A, Doumoutier L, Kotenko S, Roth JA and **Chada S**. " MDA-7/ IL-24 is a novel ligand that regulates angiogenesis via the IL-22 receptor". *Cancer Research*. 63(16):5105-13. (2003)

44) Mhashilkar AM, Stewart A, Sieger K, Ito I, Saito Y, Roth JA, Ramesh R and **Chada S**. "MDA-7 Negatively Regulates the beta-Catenin and PI3K Signaling Pathways in Breast and Lung Tumor Cells". *Molecular Therapy*, 8(2):207-19 (2003)

45) **Chada S**, Mhashilkar A and Gabrilovich D. "Development of vaccines against self antigens: the p53 paradigm". *Current Opinion in Drug Discovery and Development*. Mar;6(2):169-73 (2003)

46) Fisher PB, Gopalkrishnan RV, **Chada S**, Ramesh R, Grimm EA, Rosenfeld MR, Curiel DT and Dent P. "*mda-7*/IL-24, a novel cancer selective apoptosis inducing cytokine gene". *Cancer Biology and Therapy*: 2(4):24-38 (2003)

47) Saito Y, Gopalan B, Mhashilkar AM, Roth JA, **Chada S**, Zumstein L, Ramesh R. Adenovirus-mediated PTEN treatment combined with caffeine produces a synergistic therapeutic effect in colorectal cancer cells. *Cancer Gene Therapy*; 10(11):803-13 (2003).

48) Saito Y, Swanson X, Mhashilkar AM, Oida Y, Schrock R, Branch CD, **Chada S**, Zumstein L, Ramesh R. Adenovirus-mediated transfer of the PTEN gene inhibits human colorectal cancer growth in vitro and in vivo. *Gene Therapy* ;10(23):1961-9 (2003)

49) **Chada S**, Ramesh R, Mhashilkar AM. Cytokine- and chemokine-based gene therapy for cancer *Curr Opin Mol Ther*. 5(5):463-74 (2003). .

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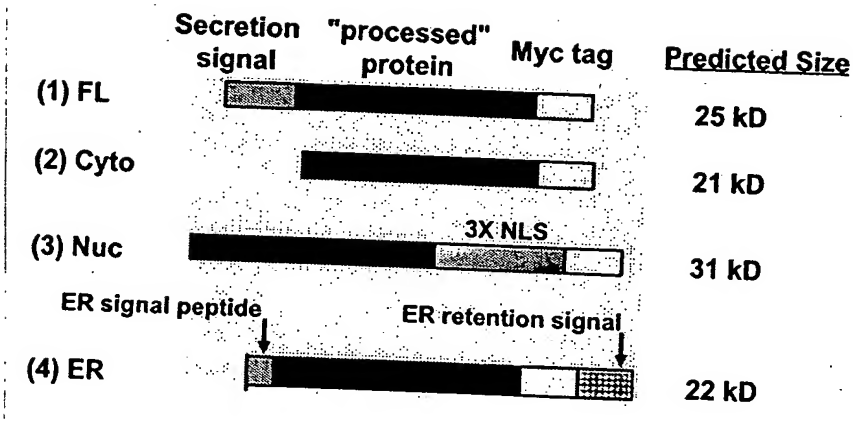
51) **Chada S**, Ekmekcioglu S, Ellerhorst JA, Mumm JB, Sahin A, Hunt KK, Ramesh R, Grimm EA and Mhashilkar AM. MDA-7/IL-24 is a unique cytokine in the IL-10 family". *International Immunopharmacology*. In Press (2004)

C. Research Support.

GRANTS AWARDED (from a total of 9)

1. Chada S. "Novel Gene Therapeutic for the Treatment of Lung Cancer" SBIR Grant 1R43CA86587-01 (Funded 03/00). Role: PI. Goals: to evaluate Ad-mda7 as a potential therapeutic for NSCLC
2. Meyn R "Tumor cell radiosensitization by gene drugs" STTR Grant. (Funded 08/00). Role: co-PI. Goal: to evaluate radiosensitization by Ad-p16 and Ad-mda7
3. Grimm EA "Novel gene therapy for Melanoma" STTR grant (Funded 06/01). Role: co-PI. Goals: to evaluate Ad--mda7 as a potential therapeutic for melanoma.
4. Chada S "Combination treatment for breast cancer using Ad-mda7 plus Herceptin". SBIR grant (Funded 07/02). Role: PI. Goals: To evaluate synergy between Ad-mda7 and Herceptin in breast cancer
5. Grimm EA "Phase II clinical trial for Melanoma using INGN 241 (Ad-mda7)" STTR grant (Funded 09/03). Role: co-PI

PATENTS and APPLICATIONS 8 issued patents; 17 applications pending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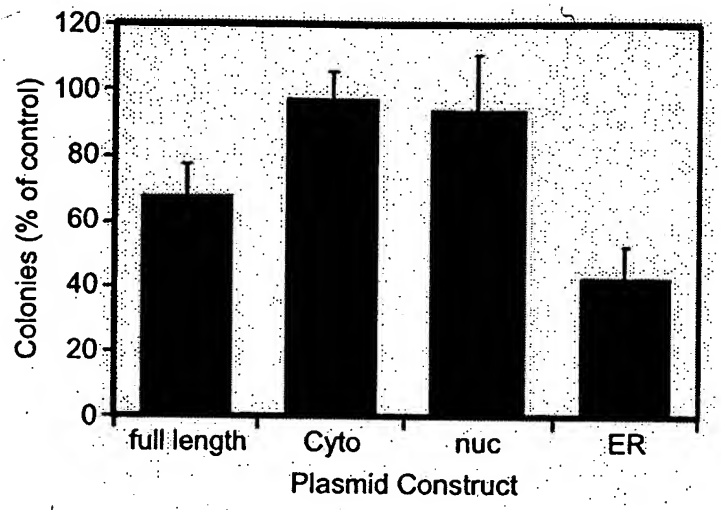
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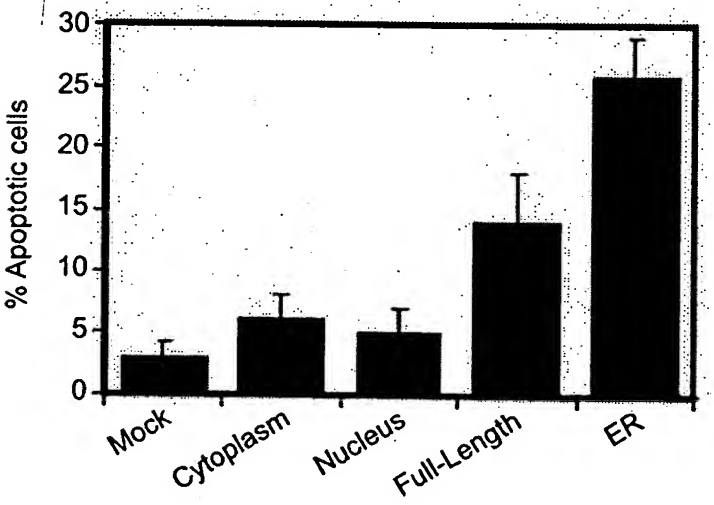
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JOURNAL OF VIROLOGY, May 2000, p. 4244–4252
0022-538X/00/$04.00+0

Vol. 74, No. 9

A Single Intramuscular Injection of Recombinant Plasmid DNA Induces Protective Immunity and Prevents Japanese Encephalitis in Mice

GWONG-JEN J. CHANG,* ANN R. HUNT, AND BRENT DAVIS

Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, U.S. Department of Health and Human Services, Fort Collins, Colorado 80522

Received 10 November 1999/Accepted 1 February 2000

Plasmid vectors containing Japanese encephalitis virus (JEV) premembrane (prM) and envelope (E) genes were constructed that expressed prM and E proteins under the control of a cytomegalovirus immediate-early gene promoter. COS-1 cells transformed with this plasmid vector (JE-4B clone) secreted JEV-specific extracellular particles (EPs) into the culture media. Groups of outbred ICR mice were given one or two doses of recombinant plasmid DNA or two doses of the commercial vaccine JEVAX. All mice that received one or two doses of DNA vaccine maintained JEV-specific antibodies 18 months after initial immunization. JEVAX induced 100% seroconversion in 3-week-old mice; however, none of the 3-day-old mice had enzyme-linked immunosorbent assay titers higher than 1:400. Female mice immunized with this DNA vaccine developed plaque reduction neutralization antibody titers of between 1:20 and 1:160 and provided 45 to 100% passive protection to their progeny following intraperitoneal challenge with 5,000 PFU of virulent JEV strain SA14. Seven-week-old adult mice that had received a single dose of JEV DNA vaccine when 3 days of age were completely protected from a 50,000-PFU JEV intraperitoneal challenge. These results demonstrate that a recombinant plasmid DNA which produced JEV EPs in vitro is an effective vaccine.

Japanese encephalitis (JE) is a mosquito-borne viral disease of major public health importance in Asia. More than 35,000 cases and 10,000 deaths are reported annually (52). *Japanese encephalitis virus* (JEV) is a member of the genus *Flavivirus* in the family *Flaviviridae*. More than 70 species in the *Flavivirus* genus have been genetically and serologically classified (29). Other important human pathogenic flaviviruses include yellow fever, dengue type 1 to 4 (DEN1 to DEN4), tick-borne encephalitis (TBE), and St. Louis encephalitis (SLE) viruses. Vaccination has been an effective mechanism for prevention of flavivirus infection in humans and domestic animals. Three JEV vaccines are in widespread production and use (52). These are inactivated virus from infected mouse brain, inactivated virus from primary hamster kidney cells, and a live attenuated SA14-14-2 vaccine. Only inactivated JEV vaccine, JEVAX, produced in mouse brain is distributed commercially and available internationally (52). Inactivated, mouse brain-derived whole virus vaccine is costly to prepare and carries the risk of allergic reaction to murine encephalitogenic basic proteins or gelatin stabilizer (45; M. M. Andersen, and T. Ronne, Letter, Lancet 337:1044, 1991). Since 1989, an unusual number of systemic reactions characterized by generalized urticaria and/or angioedema following JEVAX immunization have been reported from Australia, Canada, and Denmark (36). A major problem associated with use of the inactivated mouse brain vaccine is the failure to stimulate long-term immunity (39). Multiple immunization is recommended to provide adequate protection (28, 39). The attenuated JEV vaccine, SA14-14-2, is undergoing clinical trials (31). However, because of regulatory issues this vaccine has not found wide acceptance outside the People's Republic of China (11).

* Corresponding author. Mailing address: P.O. Box 2087, Division of Vector-Borne Infectious Diseases, CDC, Foothill Campus, Fort Collins, CO 80522-2087. Phone: (970) 221-6497. Fax: (970) 221-6476. E-mail: gxc7@cdc.gov.

Several experimental recombinant virus, attenuated virus, and subunit JEV vaccines have been reported. Recombinant baculovirus vector that contained the JEV envelope (E) protein gene has been used to infect insect cells and produce E protein that has been studied as a biosynthetic immunogen (33). Recombinant vaccinia viruses expressing the JEV genes extending from premembrane (prM) to NS2B proteins have been the most promising candidate vaccines. These candidate vaccines produced extracellular virus-like particles (EPs) in infected cell culture that induced high titers of neutralizing and hemagglutination-inhibiting antibodies and protective immunity in mice (19–21, 47, 54). Recombinant vaccinia viruses expressing the same JEV genes based on the attenuated vaccinia virus strain, NYVAC-JEV, or canarypox, ALVAC-JEV, were tested in phase I human trials (18). In this trial, only 1 in 10 ALVAC-JEV recipients developed detectable viral neutralizing antibody, and vaccinia virus-preimmune recipients had a significantly lower humoral immune response.

Inoculation of animals with purified plasmid vectors (DNA) by the intramuscular (i.m.) or intradermal route leads to expression of the recombinant vector-encoded protein in transfected cells, resulting in stimulation of a protein-specific immune response. Plasmid DNA vaccines provide an alternative to attenuated, inactivated, or virus-vectored subunit vaccines. Flavivirus DNA vaccines for Murray Valley encephalitis, DEN2, JE, SLE, and TBE (Central European encephalitis and Russian spring summer encephalitis) viruses have been developed and tested in the mouse model (4, 17, 24, 30, 38, 49). All of these plasmid DNA constructs contained similar transcriptional regulatory elements and a flavivirus gene cassette. Vaccination of mice with these plasmid DNA vaccines induced a virus-specific antibody response, as detected by enzyme-linked immunosorbent assay (ELISA). However, production of neutralizing antibody leading to 100% protection of vaccinated animals from virus challenge was observed only after multiple immunizations or delivery of DNA to the epidermis by particle

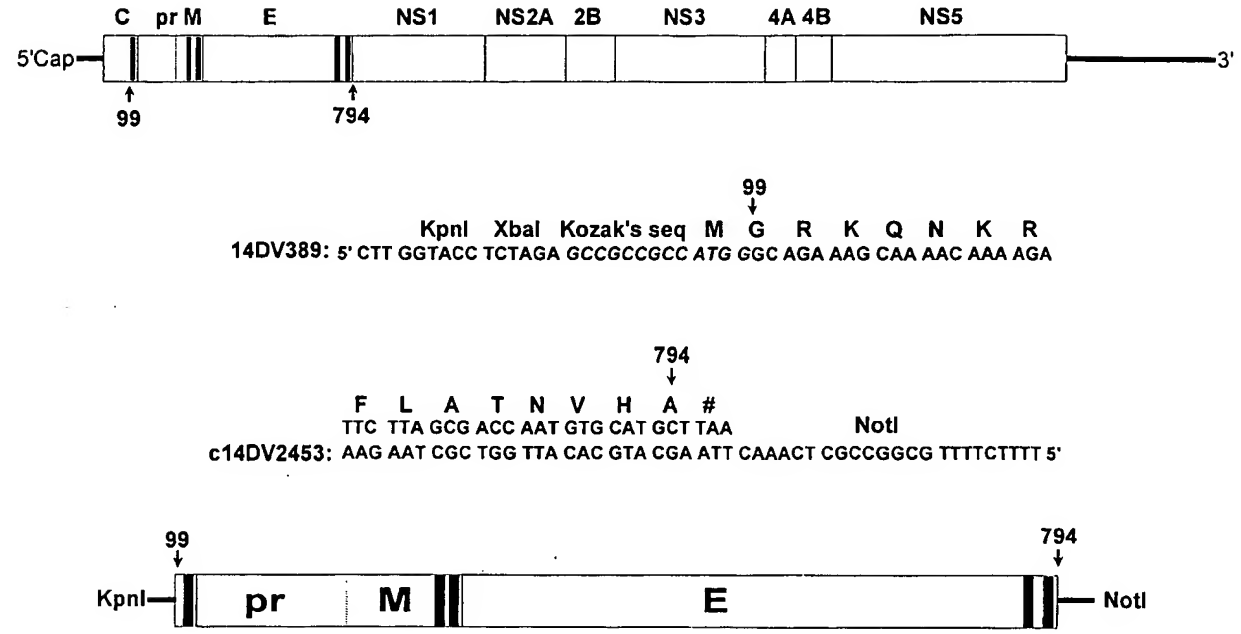


FIG. 1. Map of the JEV genomic structure (top) and the DNA sequence of oligonucleotides used in RT-PCR to construct the transcription unit for the expression of prM-E protein coding regions (bottom). Potential transmembrane helices of viral polyprotein are indicated by blackened areas.

bombardment (4, 24, 49). In this study, we constructed a JEV prM and E gene cassette that incorporates an extended signal peptide sequence at the NH_2 terminus of the prM gene and Kozak's sequence, an optimal translation enhancing element surrounding the AUG site. JEV protein expression was characterized using six different recombinant vectors containing the same insert. The humoral immune response and protection from virulent JEV challenge following immunization with the recombinant plasmid DNAs were compared to findings for the human vaccine, JEVAX, licensed by the U.S. Food and Drug Administration, in outbred ICR mice.

MATERIALS AND METHODS

Cell culture and virus strain. COS-1, COS-7, and SV-T2 cells (1650-CRL, 1651-CRL, and 163.1-CCL; American Type Culture Collection) were grown at 37°C in Dulbecco's modified Eagle medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 7.5% $NaHCO_3$ (30 ml/liter), penicillin (100 U/ml), streptomycin (100 μg/ml). COS-1 and COS-7 cells were derived from simian virus 40 (SV40) transformed CV1 cells which have an African green monkey kidney cell origin. SV-T2 cells were derived from SV40-transformed mouse fibroblasts. Vero cells were grown under the same conditions except that 5% fetal calf serum without nonessential amino acid was used. C6/36 cells (13) were grown at 28°C in the same medium used for the COS-1 cells. The SA14 strain of JEV, propagated by intracranial inoculation into suckling mouse brain, was used for animal challenges and plaque reduction neutralization tests (PRNT). The SA14 virus used in ELISA and Western blot experiments was propagated in C6/36 cells and purified by ultracentrifugation on 30% glycerol–45% potassium tartrate gradients (37).

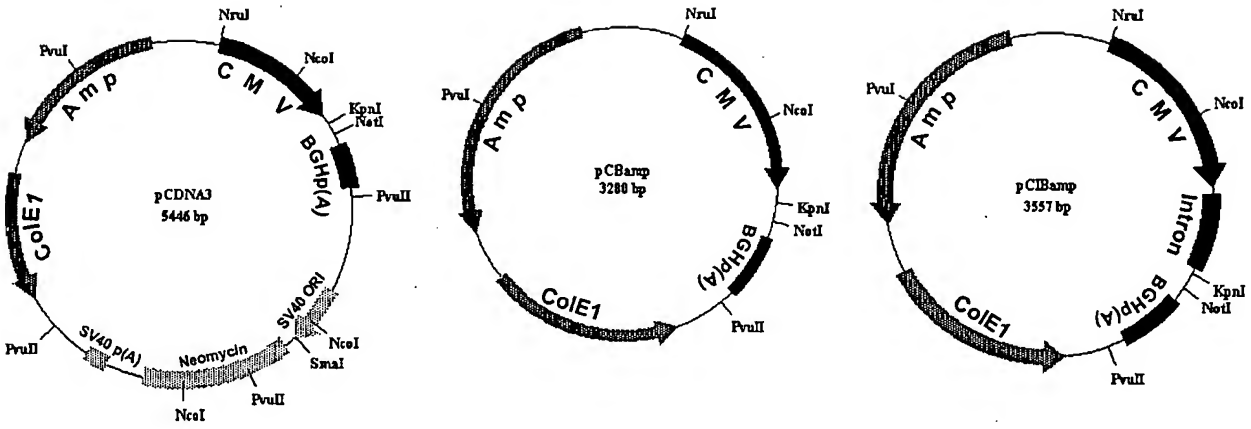
Construction of plasmids expressing JEV prM and E gene proteins. Genomic RNA was extracted from 150 μl of SA14 mouse brain JEV by using a QIAamp viral RNA kit (Qiagen, Santa Clarita, Calif.). RNA was adsorbed on a silica membrane, eluted in 80 μl of diethyl pyrocarbonate (Sigma Chemical Co., St. Louis, Mo.)-treated water, and used as a template for amplification of JEV prM and E genes. Primer sequences were obtained from the published data (35). A single cDNA fragment containing genomic nucleotides (nt) 389 to 2478 was amplified by reverse transcriptase-mediated PCR (RT-PCR). Restriction enzyme sites for *Kpn*I and *Xba*I and Kozak's sequence for an optimal translation initiation (25, 26) were engineered at the 5′ terminus of the cDNA by amplimer 14DV389. An in-frame translation termination codon, followed by a *Not*I restriction site, was introduced at the 3′ terminus of the cDNA by amplimer c14DV2453 (Fig. 1). A single-tube RT-PCR was performed using a Titan RT-PCR Kit (Roche Molecular Biochemical, Indianapolis, Ind.). The RT-PCR product was purified using a QIAquick PCR purification kit (Qiagen), and the DNA was eluted with 50 μl of 1 mM Tris-HCl (pH 7.5).

All vector constructions and analyses were carried out using standard techniques (46). RT-PCR-amplified cDNA was digested with enzymes *Kpn*I and *Not*I and inserted into the *Kpn*I-*Not*I site of eukaryotic expression plasmid vector pCDNA3 (Invitrogen, Carlsbad, Calif.). Electroportion-competent *Escherichia coli* XL1-Blue cells (Stratagene, La Jolla, Calif.) were transformed by electroporation (Gene Pulser; Bio-Rad Laboratories, Hercules, Calif.) and plated on Luria broth (LB) agar plates that contained carbenicillin (100 μg/ml; Sigma). Clones were picked and inoculated into 3 ml of LB containing carbenicillin (100 μg/ml). Plasmid DNA was extracted from a 14-h LB culture by using a QIAprep Spin Miniprep kit (Qiagen). Automated DNA sequencing was performed as recommended on an ABI Prism 377 DNA sequencer (Perkin-Elmer/Applied Biosystems, Foster City, Calif.). Both strands of the cDNA were sequenced and compared to the published SA14 virus sequence (35).

The pCDNA3 fragment from nt 1289 to nt 3455, which contained the f1-encoded eukaryotic origin of replication (ori), SV40 ori, neomycin coding region, and SV40 poly(A) elements, was deleted by *Pvu*II digestion and then self-ligated to generate plasmid pCBamp. The pCIBamp vector, which contained a chimeric intron insertion at the *Nco*I-*Kpn*I site of the pCB vector, was constructed by excising the intron sequence from pCI (Promega, Madison, Wis.) by digestion with *Nco*I and *Kpn*I. The resulting 566-bp fragment was cloned into *Nco*I-*Kpn*I-digested pCBamp to replace its 289-bp fragment. Figure 2 shows a schematic drawing of plasmids pCDNA3, pCBamp, and pCIBamp.

The DNA fragment containing the JEV coding region in the recombinant plasmid pCDJE2-7, derived from the pCDNA3 vector, was excised by *Not*I and *Kpn*I or *Xba*I digestion and cloned into the *Kpn*I-*Not*I sites of pCB, pCIB, pCEP4 (Invitrogen), and pREP4 (Invitrogen) and into the *Spe*I-*Not*I site of the pRc/RSV (Invitrogen) expression vector to create pCBJE1-14, pCIBJES14, pCEJE, pREJE, and pRCJE, respectively. Both strands of the cDNA from each plasmid vector were sequenced, and recombinant clones with a correct nucleotide sequence were identified. Plasmid DNA for in vitro transformation or mouse immunization was purified by anion-exchange chromatography using an Endo-Free Plasmid Maxi kit (Qiagen).

IFA. Expression of JEV-specific gene products by the various recombinant expression plasmids was evaluated by indirect immunofluorescence antibody assay (IFA) in the transient expression system using COS-1, COS-7, and SV-T2 cells. For transformation, cells were grown to 75% confluence in 150-cm^2 culture flasks, trypsinized, and resuspended in 4°C phosphate-buffered saline (PBS) to a final density of 1×10^7 to 2×10^7 cells/ml. Five hundred microliters of cell suspension was then electroporated with 10 μg of plasmid DNA, using a Bio-Rad Gene Pulser II set at 250 V and 960 μF. Cells were diluted with 25 ml of fresh medium after electroporation and seeded into one 75-cm^2 flask. Forty-eight hours after transformation, the medium was removed, and the cells were



TATA box KpnI BamHI EcoRI EcoRV NotI

TATATAA...(92nt)...*GGTACC*GAGCTC*GGATCC*ACTAGTAACGGCCGCCAGTGTGCTG*GAATTC*TGCA*GATATC*CATCACACTG*GCGGCCGC*...BHG-poly(A)

FIG. 2. Schematic representations of plasmid vectors pCDNA3, pCBamp, and pCIBamp. These plasmids include the CMV promoter/enhancer element, BGH poly(A) signal and transcription termination sequence [BHGp(A)], ampicillin resistance gene (Amp), and ColE1 ori for selection and maintenance in *E. coli*. The f1 ori for single-stranded rescue in *E. coli* cells, SV40 ori, neomycin coding region, and SV40 poly(A) [SV40 p(A)] sequences were deleted from pCDNA3 to generate pCBamp. An intron sequence was inserted in the *NcoI-KpnI* site of pCBamp to generate pCIBamp. The multiple cloning site for the insertion of JEV genes, located between the TATA box of the CMV promoter/enhancer and BHG poly(A) site, is shown.

trysinized and resuspended in 5 ml of PBS with 3% normal goat serum. Ten-microliter aliquots of the cell suspension were then spotted onto slides, air dried, and fixed with acetone at 4°C for 10 min. Immunofluorescent mapping of the E protein-specific epitopes was performed using a panel of murine monoclonal antibodies (MAbs) (15, 42, 55) and JEV-specific hyperimmune mouse ascitic fluid (HIAF). All antibodies were tested at 1:400 dilution in PBS.

Selection of an in vitro-transformed stable cell line constitutively expressing JEV-specific gene products. COS-1 cells transformed with 10 μg of pCDJE2-7 DNA by electroporation were incubated in nonselective culture medium for 24 h and then treated with neomycin (G418; 0.5 mg/ml; Sigma). G418-resistant colonies, which became visible after 2 to 3 weeks, were cloned by limited dilution in G418-containing medium. Expression of the JEV proteins was determined by IFA using JEV HIAF. One IFA-positive (JE-4B) and one IFA-negative (JE-5A) clone were selected for further analysis and maintained in medium containing 200 μg of G418 per ml. These stably transformed cells secreted antigen in the form of EPs (A. Hunt and G. J. Chang, unpublished data).

Antigen capture ELISA for detection of E protein secreted into culture fluid. The antigen capture ELISA, a modification of the procedure described by Guirakhoo et al. (8), was used to detect E protein from transiently transformed cells or JE-4B culture fluid. Flavivirus group-reactive MAb 4G2 was used to capture the JEV antigens (7). The 4G2-captured antigen was detected using horseradish peroxidase-conjugated MAb 6B6C-1 by incubation for 1 h at 37°C. Enzyme activity on the solid phase was detected with 3,3',5,5'-tetramethylbenzidine ELISA substrate (Life Technologies, Grand Island, N.Y.); the reaction was stopped with the addition of 2 M H_2SO_4, and the optical density was measured at 450 nm.

Mouse experiments. Three-day-old mixed-sex or 3-week-old female ICR outbred mice were vaccinated i.m. with 50 or 100 μg of plasmid DNA at a concentration of 1 μg/μl in PBS or subcutaneously (s.c.) with 1/10 or 1/5 of the adult human dose of JEVAX (manufactured by the Research Foundation for Microbial Disease of Osaka University and distributed by Connaught Laboratories, Swiftwater, Pa.). The chloramphenicol acetyltransferase (CAT) protein expression plasmid pCDNA3/CAT (Invitrogen) was used as the vaccination control. Selected groups of mice were boosted 3 weeks later with an additional dose of plasmid vaccine or JEVAX. Mice were bled from the retro-orbital sinus; serum samples were evaluated for JEV antibody by ELISA and Western blotting using purified JEV and by PRNT.

Mice vaccinated at 3 days of age were challenged intraperitoneally (i.p.) 7 weeks postvaccination with JEV strain SA14 (50,000 PFU/100 μl) and observed for 3 weeks. To evaluate passive protection by maternal antibody, pups were obtained from mating of nonimmunized males with immunized females 9 weeks following their vaccination with plasmid DNA at 3 weeks of age. Pups were challenged by the i.p. route 3 to 15 days after birth with SA14 virus (5,000 PFU/100 μl) and observed daily for 3 weeks. Postchallenge serum was collected from survivors and tested for reactivity with JEV antigens by ELISA and Western blotting.

Serological tests. Postvaccination and postchallenge serum samples were tested for the ability to bind to purified JEV by ELISA, neutralize JEV infectivity by PRNT, or recognize JEV proteins by Western blotting (12, 41, 48). The PRNT assay was performed by incubating ~200 PFU of SA14 virus in 100 μl of Dulbecco's modified Eagle medium containing 5% bovine serum albumin and 20 mM HEPES buffer (pH 8.0) with serial twofold dilutions of serum specimens, started at 1:10, in 100 μl of the same buffer in 96-well trays at 4°C overnight. Serum specimens were heat inactivated at 56°C for 30 min before use. Duplicate 100-μl aliquots were assayed for infective virus by plaque formation on Vero cell monolayers. The percent plaque reduction was calculated relative to virus controls without serum. Titers were expressed as the reciprocal of serum dilutions yielding a 90% reduction in plaque number ($PRNT_{90}$).

RESULTS

Effect of the promoter and poly(A) signal on the efficiency of JEV prM and E protein expression. Four eukaryotic cell expression plasmids that contained the JEV coding region extending from genomic nt 390 to nt 2478 were constructed. This region of the genome encoded the prM and E genes. The Kozak sequence for the eukaryotic translation initiation site (underlined) of −9 to +4, GCCGCCGCC<u>ATG</u>G, at the 5′ terminus (2, 25, 26, 27) and the in-frame translation terminat ion sequence at the 3′ terminus of cDNA were incorporated directly into cDNA by RT-PCR using viral RNA as a template. Transcription of the JEV genes in plasmid pCDJE2-7 was controlled by the human cytomegalovirus (CMV) early IA gene promoter/enhancer. The resulting mRNA is terminated and stabilized by a bovine growth hormone (BGH) transcript ion terminator and a poly(A) signal, respectively. The transcriptional control elements in pREJE were replaced by the Rous sarcoma virus (RSV) long terminal repeat promoter and SV40 poly(A). The pCEJE and pRCJE plasmids contain CMV plus SV40 poly(A) and RSV plus BGH poly(A), respectively (Table 1).

To determine the influence of the promoter and poly(A) elements on JEV prM and E protein expression, recombinant plasmids pCDJE2-7, pCEJE, pRCJE, and pREJE were ini-

TABLE 1. Transient expression of JEV prM and E proteins by various recombinant plasmids in two transformed cell lines

Name	Promoter	Intron	Poly(A)	Ori	Recombinant plasmid	IFA intensity/% positive[a]	
						COS-1	COS-7
pCDNA3	CMV	No	BGH	SV40	pCDJE2-7	3+/40	3+/35
pCBamp	CMV	No	BGH	No	pCBJE1-14	3+/45	ND
pCIBamp	CMV	Yes	BGH	No	pCIBJES14	3+/39	ND
pCEP4	CMV	No	SV40	OriP	pCEJE	2+/4	2+/3
pREP4	RSV	No	SV40	OriP	pREJE	1+/3	1+/2
pRc/RSV	RSV	No	BGH	SV40	pRCJE	1+/3	1+/3
pCDNA3	CMV	No	BGH	SV40	pCDNA3/CAT	−	−

[a] Various cell lines were transformed with pCDNA3/CAT (negative control), pCDJE2-7, pCBJE1-14, pCIBJES14, pCEJE, pREJE, or pRCJE. Cells were trypsinized 48 h later and tested by IFA with JEV HIAF. Data are presented as the intensity (scale of 1+ to 4+) and percentage of IFA-positive cells. pCDNA3/CAT-transformed cells were used as the negative control. ND, not determined. −, negative.

tially tested for the ability to express JEV prM and E proteins following transformation of various mammalian cells. COS-1, COS-7, and SV-T2 cells were transiently transformed with equal amounts of pCDJE2-7, pCEJE, pRCJE, or pREJE plasmid DNA. The SV-T2 cell line was excluded from further testing after preliminary results showed that less than 1% of pCDJE2-7-transformed SV-T2 cells were expressing JEV antigen.

JEV antigens were expressed in COS-1 and COS-7 cells transformed by all four recombinant plasmids, thus confirming that the CMV or RSV promoter and BGH or SV40 poly(A) elements were functionally active. However, the percentage of transformed cells and the level of JEV antigens expressed, as determined by the number of IFA-positive cells and IFA intensity, respectively, differed significantly (Table 1). A significantly higher percentage of pCDJE2-7-transformed COS-1 cells expressed JEV proteins with greater IFA intensity at a level equal to that observed with JEV-infected cells. Cells transformed with the pCEJE, pREJE, or pRCJE vector, on the other hand, showed a lower percentage of antigen-expressing cells as well as a lower IFA intensity. Vectors containing the CMV promoter and BGH poly(A) were selected for further analysis (Fig. 2).

To determine whether the enhanced expression of JEV proteins by the pCDJE2-7 vector was influenced by the SV40 ori, we constructed the pCBJE1-14 vector in which a 2,166-bp fragment containing the f1 ori, SV40 ori, neomycin coding region, and SV40 poly(A) elements was deleted. A chimeric intron was then inserted into pCBJE1-14 to generate pCIBJES14. Plasmid pCIBJES14 was used to determine whether the expression of JEV proteins could be enhanced by an intron sequence. Following transformation, both pCBJE1-14 and pCIBJES14 vectors resulted in cells expressing levels of JEV proteins similar to that observed with the pCDJE2-7 vector (Table 1). These results indicated that expression of the JEV proteins was influenced only by the transcriptional regulatory elements encoded in the recombinant plasmid. Neither the SV40 ori nor the intron sequence enhanced JEV protein expression in the cells used.

Epitope mapping of E protein expressed by a stably transformed cell line constitutively expressing JEV-specific gene products. Authenticity of the JEV E protein expressed by the JE-4B clone was demonstrated by epitope mapping by IFA using a panel of JEV E-specific murine MAbs. JEV HIAF and one irrelevant mouse ascitic fluid were used as positive and negative antibody controls, respectively. Four JEV-specific, six flavivirus subgroup-specific, and two flavivirus group-reactive MAbs reacted similarly with the 4B clone and with JEV-infected COS-1 cells (Table 2).

Detection of JEV E protein secreted by the JE-4B COS-1 cell line. An antigen capture ELISA, employing flavivirus group-reactive, anti-E MAbs 4G2 and 6B6C-1, was used to detect JEV E proteins that were secreted into the culture fluid by the COS-1 cell clone JE-4B. Antigen could be detected in the culture fluid the first day following seeding of the cells with maximum ELISA titers that ranged from 1:16 to 1:32.

Comparison of immune responses in mice vaccinated with pCDJE2-7 genetic vaccine and JEVAX. Plasmid pCDJE2-7 was used as a nucleic acid vaccine to induce an antibody response in mice by immunizing groups of five 3-week-old female ICR outbred mice. Mice were bled at 3, 6, 9, 23, 40, and 60 weeks after immunization, and antibody titers were determined by ELISA or by PRNT. As expected, sera from animals in the pCDNA3/CAT control group did not contain JEV antibody. All animals immunized with pCDJE2-7 and JEVAX seroconverted by 3 weeks after the first vaccination (Table 3). The antibody titers were similar irrespective of the number of doses

TABLE 2. Epitope mapping of E protein expressed by JE-4B, a pCDJE2-7 stably transformed clone of COS-1 cells, with JEV-reactive antibodies[a]

MAb or antiserum	Biological activity of MAb		IFA intensity of cells	
	Specificity	Biological function	JEV infected	4B
MAbs				
MC3	JEV specific		2+	2+
2F2	JEV specific	HI, N	4+	4+
112	JEV specific		4+	4+
503	JEV specific	N	4+	3+
109	Subgroup	HI	2+	1+
N.04	Subgroup	HI, N	3+	4+
201	Subgroup		1+	1+
203	Subgroup		4+	3+
204	Subgroup		2+	2+
301	Subgroup	HI	2+	2+
504	Flavivirus		4+	4+
6B6C-1	Flavivirus		2+	2+
3B4C-4	VEE		−	−
HIAF				
Anti-JEV			4+	3+
Anti-WEE			−	−
PBS			−	−

[a] VEE, Venezuelan equine encephalomyelitis virus; WEE, Western equine encephalomyelitis virus. −, negative.

TABLE 3. Persistence of the immune response in mice (five per group) immunized with pCDJE2-7 or JEVAX

Inoculation[a]	ELISA titer (log_{10})						$PRNT_{90}$ titer		
	3[b]	6	9	23	40	60[c]	3	6	9
pCDJE2-7									
1 dose	2.6–3.2	3.8–5.0	3.8–4.4	>3.2	>3.2	2.4, 2.4, 3.8, 4.4	<20	20	40–160
2 doses	2.6–3.8	4.4	3.8–4.4	>3.2	>3.2	2.6, 3.8, 3.8	<20	20–40	40–160
JEVAX, 2 doses	2.6–3.8	4.4–5.0	3.8–5.6	>3.2	>3.2	<2, <2, <2, 4.4	<20	20–40	20–160
pCDNA3/CAT, 2 doses	<100	<100	<100	ND[d]	ND	ND	<20	<20	<20

[a] Three-week-old mice were inoculated i.m. with one or two 100-μg doses of plasmid DNA or twice s.c. with one-fifth of the human dose of JEVAX.
[b] Weeks postimmunization.
[c] Individual serum titers.
[d] ND, not determined.

of pCDJE2-7 or JEVAX given. Mouse serum samples collected 9 weeks after immunization were also tested by Western blotting using purified JEV. Serum specimens from DNA-vaccinated mice, which had reactivity similar to that of JEV HIAF, detected E and prM proteins (Fig. 3). However, mouse serum from JEVAX-immunized mice reacted only with E protein. Comparable ELISA antibody titers were maintained in DNA-vaccinated groups for up to 60 weeks, at which time the experiment was terminated. Only one of four mice in the JEVAX group remained JEV antibody positive at 60 weeks postinoculation. These results demonstrated that one dose of JEV-specific nucleic acid vaccine was more effective in maintaining JEV antibody levels in mice than the commercially available vaccine JEVAX.

Comparison of various nucleic acid vaccine constructs and JEVAX for ability to induce JEV-reactive antibody in different age groups of mice. Similar amounts of JEV protein were expressed by COS-1 cells transformed by either pCDJE2-7, pCBJE1-14, or pCIBJES14. JEV antibody induction by these nucleic acid constructs was compared to results for JEVAX in two different age groups of mice. Three-day-old mixed-sex or 3-week-old female ICR outbred mice, 10 per group, were vaccinated i.m. with 50 or 100 μg of plasmid DNA or s.c. with 1/10 or 1/5 of the adult human dose of JEVAX, respectively. Serum specimens were collected at 7 weeks after immunization and tested at 1:400 or 1:1,600 by ELISA. Ninety to 100% of all 3-week-old mice that received pCBJE1-14, pCDJE2-7, pCIBJES14, or JEVAX had antibody titers of ≥1:1,600. However, a significant difference in antibody response was observed in 3-day-old groups that received various vaccines. None of the 3-day-old JEVAX-vaccinated mice had antibody titers higher than 1:400. All 3-day-old mice vaccinated with pCBJE1-14 had antibody titers higher than 1:1,600. Seroconversion of 100% was observed at 1:400 in 3-day-old mice that received pCDJE2-7 or pCIBJES14, but only 60% of both mouse groups were positive at 1:1,600. pCBJE1-14 was the most effective of three DNA constructs tested. The minimum dose of this DNA construct capable of providing 100% seroconversion (1:400 by ELISA) by i.m. immunization in 3-week-old mice was determined to be 25 μg (data not shown).

Protective immunity conferred by the nucleic acid vaccine. Mice immunized at 3 days of age were challenged by the i.p. route at 7 weeks postvaccination with the SA14 strain of JEV (50,000 PFU/100 μl) and observed for 3 weeks. One hundred percent of the animals that received various nucleic acid vaccine constructs were protected. In contrast, only 40 and 30% of mice that received JEVAX and pCDNA3/CAT, respectively, survived virus challenge (Fig. 4). These results suggested that the DNA vaccine could be effective as a neonatal vaccine. In contrast, JEVAX was not as effective in neonatal animals.

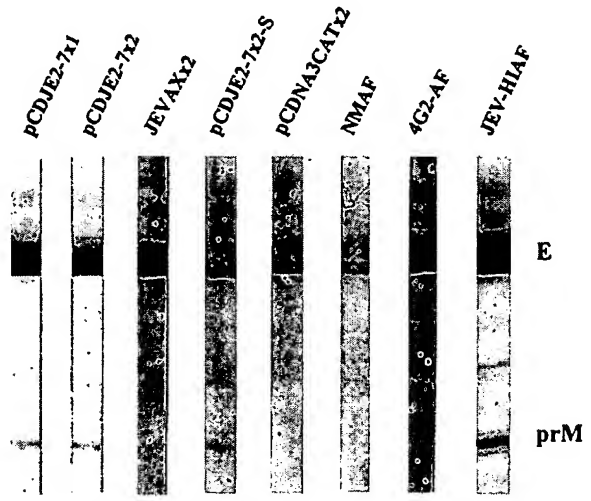


FIG. 3. JEV-specific reactivity of prechallenge and postchallenge serum samples obtained from mice immunized with DNA vaccine or JEVAX. Serum specimens collected from the mice used in the experiments represented in Tables 3 and 4 were randomly selected and tested at 1:1,000 dilution by Western blot analysis using purified JEV as the antigen. pCDJE2-7x2-S was the serum from one of the mice challenged at 4 days of age (Table 4). NMAF, 4G2-AF, and JEV HIAF were the mouse ascitic fluids included as normal mouse, E-specific, and JEV hyperimmune controls, respectively.

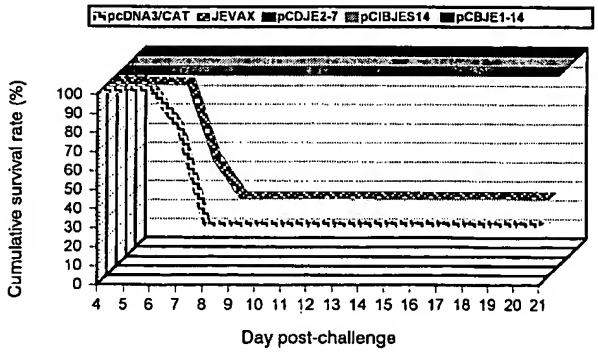


FIG. 4. Postchallenge survival rates of mice (10 per group) that were immunized with pCDJE2-7, pCBJE1-14, pCIBJES14, pcDNA3/CAT, or JEVAX at 3 days of age and challenged i.p. with 50,000 PFU of JEV (SA14) 7 weeks postimmunization. A P value of 0.003 was obtained by Fisher's exact test when the survival rate of the JEV DNA-immunized groups was compared with that of the pcDNA3/CAT or JEVAX group.

TABLE 4. Ability of maternal antibody from JEV nucleic acid-vaccinated female mice to protect their pups from fatal JE

Vaccinated mother[a]		JEV-challenged pups			
Vaccine	$PRNT_{90}$	Age (days)	No. of survivors/ total in litter	Avg survival time (days)	ELISA[b]
1 × pCDJE2-7	40	4	0/11	5.27	
2 × pCDJE2-7	80	4	12/12	NA[c]	12/12
2 × JEVAX	20	3	0/16	4.75	
2 × pCDNA3/CAT	<10	5	0/14	4.00	
1 × pCDJE2-7	20	15	5/11	10.0	5/5
2 × pCDJE2-7	40	14	8/12	13.75	7/8
2 × JEVAX	80	13	5/5	NA	5/5
2 × pCDNA3/CAT	<10	14	0/14	6.14	

[a] Mice were inoculated i.m. with one or two 100-μg doses of pCDJE2-7 DNA or twice s.c. with one-fifth of the adult human dose of JEVAX. Serum samples were collected 9 weeks postvaccination for PRNT testing prior to mating with nonimmune male.

[b] Number of JEV ELISA antibody-positive animals (titer ≥ 1:400)/number of survivors. Serum specimens were collected for testing 12 weeks after challenge.

[c] NA, not applicable.

Passive protection of neonatal mice correlated with the maternal antibody titer. Female 3-week-old ICR mice were vaccinated with one or two doses of pCDJE2-7 plasmid DNA (100 μg/100 μl) or twice with one-fifth of the adult human dose of JEVAX. For evaluation of passive protection by maternal antibody, pups were obtained from matings of experimental females with nonimmunized male mice. Pups were challenged by the i.p. route at 3 to 5 or 13 to 15 days after birth with SA14 virus (5,000 PFU/100 μl). Survival rates and average survival time correlated with the maternal neutralizing antibody titers (Table 4). One hundred percent of pups nursed by mothers with a PRNT of 1:80 survived viral infection regardless of the type of vaccine received by the mothers. None of the pups from mothers which received pCDNA3/CAT plasmid DNA survived (Table 4). Partial protection (45% [5 of 11 pups] to 67% [8 of 12 pups]) was observed in older pups that were nursed by the mothers which had serum PRNT titers of 1:20 and 1:40, respectively. However, none of the 3-day-old pups survived virus challenge when the mothers had a serum PRNT titer of 1:20 or 1:40. Maternally transferred antibody can only be detected in the circulation of the young mouse up to 40 days after birth. An appreciable level of maternally derived antibody is maintained in the circulation of the young mouse 24 days or more postpartum (1). JEV ELISA antibody detected in the serum of 97% (29 of 30) of the postchallenge pups at 12 weeks after virus challenge was unlikely to be residual maternally transferred antibody. The presence of JEV antibody in the surviving pups challenged at 3 to 4 or 13 to 15 days of age strongly suggested that maternal antibody did not provide sterilizing immunity to the pups. It also indicated that 3- to 4- or 13- to 15-day-old mice could mount an immune reaction to a live-virus challenge. Partial protection in older pups could be explained by the opportunity to accumulate a large quantity of passive antibody due to the length of nursing time before challenge. One randomly selected postchallenge serum sample also reacted with prM and E proteins by Western blotting (Fig. 3).

DISCUSSION

The flavivirus virion contains a capsid protein (C), a membrane protein (M), and an E protein. The prM MAbs, exhibiting weak or undetectable neutralizing activity in vitro, can provide passive protection following DEN2 virus challenge (16). However, the E protein plays a dominant role in generating neutralizing antibodies and providing protective immunity in the host. Passive transfer of JEV E-specific neutralizing MAbs has been shown to protect recipients from JEV-induced fatal encephalitis (3, 16, 32, 55). Antigenic and structural analysis using various panels of MAbs has shown that most of the E protein epitopes that elicit virus-neutralizing antibodies are conformationally dependent (9, 40). Coexpression of both proteins as type I transmembrane proteins is essential to maintain proper E conformation and prevent the E protein from undergoing irreversible, low-pH-catalyzed conformational changes (8–10, 19, 50). A 2-kb genomic region, from the internal signal peptide at the carboxyl terminus of C to the transmembrane domain at the carboxyl terminus of the E gene, is essential for expressing authentic proteins. These authentic prM and E proteins are able to self-assemble into virus-like particles in cells infected by either recombinant vaccinia virus or alphavirus vector or in cells transformed by recombinant plasmid DNA (4, 19, 22, 48; Hunt and Chang, unpublished data).

A gene cassette including the elements listed above was amplified from SA14 virus by RT-PCR in the present study. Optimal sequence composition surrounding the translation initiation site (−9 to +4) was incorporated into the 14DV398 amplifying primer (2, 26, 27) (Fig. 1). Recombinant plasmids containing the CMV early gene promoter/enhancer and the BHG poly(A) terminator as transcription regulatory elements expressed JEV proteins with the highest efficiency in three different cell lines. Protein expression and the serological response of mice immunized with DNA vaccine were not influenced by the presence or absence of the SV40 ori or an intron sequence in recombinant plasmids. Virus-specific proteins, secreted into culture medium, could be detected by antigen capture ELISA as early as 48 h after plasmid transformation (data not shown). The authenticity of the E protein produced by the pCDJE2-7 stably transformed cell line, JE-4B, was demonstrated by MAb epitope mapping.

Vaccine potential and characteristics of various eukaryotic plasmids that express flavivirus prM and E proteins are summarized in Tables 5 and 6. All constructs listed had the same transcriptional control elements and similar viral gene cassettes. DEN2 plasmid, which contains prM and 91% of E, is the only exception (Table 6). The JEV DNA vaccine reported in this study is the only construct that stimulated complete protective immunity in mice by a single dose of vaccine given by the i.m. route (Table 5). Sequences surrounding the translation initiation site and the composition of the signal peptide preceding the prM protein are the two major differences among the constructs that may contribute to increasing the vaccine potential of our construct (Table 6). Conserved features of the sequences which flank vertebrate translation initiation sites include a strong preference for purine at the −3 position; a higher frequency of G at positions −9, −6, −3, and +4; and a preference for A or C at positions −5, −4, −2, and −1 (2). Instead of the sequence used in previous publications, the sequence used in our construct was −9 · GCCGCCGCC ATGG, which fits the general criteria listed above. Although less than 1% of eukaryotic mRNA sequences exhibit this sequence, the experimental data have suggested that this sequence provides exceptionally high levels of translation potential (2, 26).

Signal peptides determine translocation and orientation of inserted protein, hence the topology of prM and E. Signal peptide differences in our plasmid construct may account for the efficient translocation and correct topology, thus increasing prM and E secretion. A machine-learning program using neu-

TABLE 5. Vaccine potential of various eukaryotic plasmids that express flavivirus prM and E proteins[a]

Virus	In vitro secretion of EPs	Immunization			Protection from virus challenge	Reference
		Dosage	Route/method	Neutralizing antibody[b]		
JE	Yes	25–100 μg × 1	i.m./needle	Yes ($1:20–1:160_{90\%}$)	100%	This report
	ND	100 μg × 2	i.m./needle	No	Partial	30
	ND	10–100 μg × 2	i.m. or i.d./needle	Yes ($1:10–1:20_{90\%}$)	100%	24
MVE	Yes	100 μg × 4	i.m./needle	ND	Partial	4
	Yes	1–2 μg × 2–4	i.d./gene gun	Yes ($80–320_{50\%}$)	100%	4
SLE	ND	100 μg × 2	i.m./needle	No	Partial	38
CEE	ND	1 μg × 1–2	i.d./gene gun	Yes ($1:100–1:1,600_{80\%}$)	100%	49
RSSE	ND	1 μg × 1–2	i.d./gene gun	ND	100%	49
DEN2	ND	200 μg × 3	i.d./needle	Yes ($1:10–1:320_{50\%}$)	None	17

[a] MVE, Murray Valley encephalitis; CEE, Central European encephalitis; RSSE, Russian spring-summer encephalitis; i.d., intradermal; ND, not done.
[b] Plaque reduction neutralization titer followed by percentage reduction endpoint used in the test.

ral networks trained on eukaryotes (SignalP-NN at http://www.cbs.dtu.dk/services/) was applied to test the efficiency of the prM signal peptide sequence in the different plasmid constructs (34) (Table 6). The most probable location and orientation of transmembrane helices in the prM-E protein were then determined by a hidden Markov model-trained computer program (6 [TMHMM at http://www.cbs.dtu.dk/services/]). SignalP-NN searches correctly predicted the signal peptidase cleavage site of all constructs. However, a considerable difference in cleavage potential (C score, between 0.578 and 1.000) was observed (Table 6). Cleavage potential differences may be influenced by the amino acid composition and length of the h region in various constructs (44).

The TMHMM program correctly predicted five transmembrane helices encoded in the prM-E protein. Significant difference in the probable orientation of the first transmembrane helix was observed in three JEV constructs (Fig. 5). In our pCDJE2-7 construct, the first 12 amino acids of the n region form a short loop in the cytoplasmic side that causes the following h region (transmembrane helix) to be inserted in a tail orientation. Secretion of JEV protein could be detected by antigen capture ELISA in pCDJE2-7 transient expression studies in which less than 5% of the cells were positive by IFA (data not shown). Thus, there is a high probability that prM and E proteins expressed by pCDJE2-7 would be expressed in the correct orientation, as type I transmembrane proteins (Fig. 5A). There is also a high probability that the prM protein of pcDNA3JEME could be expressed as a type II membrane protein with its transmembrane h region inserted in a head orientation because of the absence of positively charged amino acids in its n region (Fig. 5B). Efficient protein synthesis in conjunction with correct topology of expressed prM and E (Fig. 5A) would most likely enhance EP formation and secretion in transformed cells.

Another characteristic that could explain the excellent vaccine potential of our JEV construct is its ability to produce EPs which have a virus-like polymeric structure that enhances antigenic stability and provides a high-density presentation to antigen-presenting cells, such as macrophages, dendritic cells, and Langerhans cells (5). When DNA is given by the i.m. route, the majority of antigen is expressed by non-antigen-presenting muscle cells. The efficacy of a DNA vaccine is therefore dependent on transfection of antigen-presenting cells or to reprocessing of antigen derived from other cells. Muscle cells transfected by our construct could conceivably synthesize and secrete EPs, which are highly immunogenic and have been shown to elicit good cellular and humoral responses (22, 23).

Genetic JEV vaccine that induced a completely protective immunity in neonatal mice and a maternally transferable protective immunity in young adult mice by a single i.m. immunization was demonstrated in this study. Additional studies are planned to address the effectiveness of a DNA vaccine in overcoming the potential influence of maternally transferred flavivirus antibodies on the induction of JEV antibody in neonatal mice.

Immunization of pigs is a theoretical means of interrupting transmission and amplification of JEV and thereby preventing human infections (43). The JEV DNA vaccine could also be used as a veterinary vaccine in pregnant sows to prevent JEV-induced stillbirth and abortion (51, 53). Maternally transferred antibody could also interrupt piglets as the JEV-amplifying host and thus reduce human infection.

TABLE 6. Characteristics of various eukaryotic plasmids expressing flavivirus prM and E proteins

Virus[a]	Plasmid	Sequence surrounding translation initiation site	Amino acids preceding prM protein[b]	SP potential (C score)[c]	Reference
JE	pCDJE2-7	−9•GCCGCCGCCATGG•+4	MGRKQNKRGGNEGSIMWLASLAVVIACAGA /MKL	Yes (0.921)	This report
	pJME	−9•GGCTCAATCATGG•+4	MWLASLAVVIACAGA /MKL	Yes (0.578)	30
	pCDNA3JEME	−9•GAATTCACCATGG•+4	MNEGSIMWLASLAVVIACAGA /MKL	Yes (0.921)	24
MVE	pCDNA3.prM-E	−9•TGATTTCAAATGT•+4	MSKKRGGSETSVLMVIFMLIGFAAA /LKL	Yes (0.819)	4
SLE	pSLE1	?	?LDTINRRPSKKRGGTRSLLGLAALIGLASS /LQL	Yes (0.709)	38
DEN2	p1012D2ME	?	?AGMIIMLIPTVMA /FHL	Yes (0.646)	17
TBE	SV-PE_{w1}	−9•GCGGCCGCCATGG•+4	MVGLQKRGKRRSATDWMSWLLVITLLGMTLA /ATV	Yes (1.000)	48
RSSE	pWRG7077	−9•GTAGACAGGATGG•+4	MGWLLVVVLLGVTLA /ATV	Yes (0.762)	50
CEE	pWRG7077	−9•ACGGACAGGATGG•+4	MSWLLVITLLGMTIA /ATV	Yes (0.609)	50

[a] Abbreviations are as given in Table 5, footnote *a*.
[b] Single amino acid code. Positively charged amino acid is indicated by bold letter. Signal peptidase cleavage site is indicated by /.
[c] Cleavage potential of signal peptide (SP) predicted by SignalP-NN at http://www.cbs.dtu.dk/services (34).

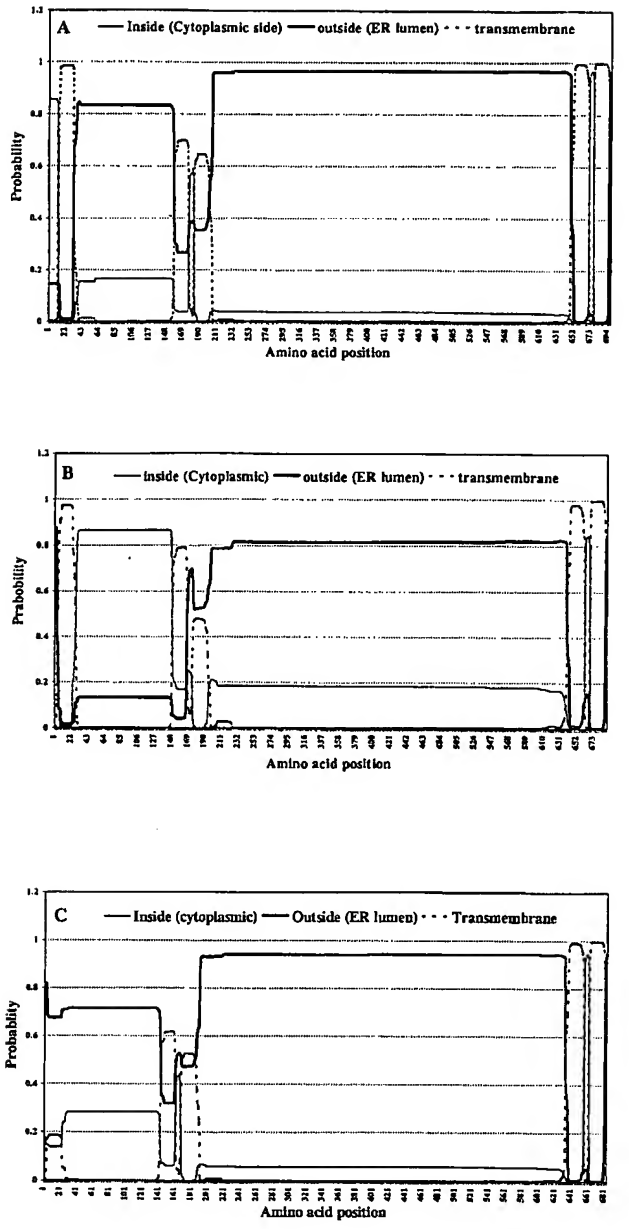


FIG. 5. Graphic representation, generated by the TMHMM program, indicating probable orientations of five transmembrane helices in the prM-E protein expressed by pCDJE2-7 (A), pcDNA3JEME (B), and pJME (C). ER, endoplasmic reticulum.

ACKNOWLEDGMENTS

We thank K. Yasui and M.-J. Zhang for providing JEV MAbs and J. Roehrig and B. Miller for useful discussion and advice. We thank D. Holmes, C. Lin, N. Frank, and T. Springfield for superb technical assistance and animal care.

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JOURNAL OF VIROLOGY, Sept. 2000, p. 8213–8217
0022-538X/00/$04.00+0

Vol. 74, No. 17

Sex Differences in Seoul Virus Infection Are Not Related to Adult Sex Steroid Concentrations in Norway Rats

SABRA L. KLEIN, BRIAN H. BIRD, AND GREGORY E. GLASS*

Department of Molecular Microbiology and Immunology, The Johns Hopkins School of Hygiene and Public Health, Baltimore, Maryland 21205-2179

Received 14 April 2000/Accepted 8 June 2000

Field studies of hantavirus infection in rodents report that a higher percentage of infected individuals are males than females. To determine whether males were more susceptible to hantavirus infection than females, adult male and female Long Evans rats (*Rattus norvegicus*) were inoculated with doses of Seoul virus ranging from 10^{-4} to 10^{6} PFU. The 50% infective doses (ID_{50}) were not significantly different for male and female rats ($10^{0.05}$ and $10^{0.8}$ PFU, respectively). To determine whether sex differences in response to infection were related to circulating sex steroid hormones, sex steroid concentrations were manipulated and antibody responses and virus shedding were assessed following inoculation with the ID_{90}. Regardless of hormone treatment, males had higher anti-Seoul virus immunoglobulin G (IgG) and IgG2a (i.e., Th1) responses than females and IgG1 (i.e., Th2) responses similar to those of females. Males also shed virus in saliva and feces longer than females. Manipulation of sex steroids in adulthood did not alter immune responses or virus shedding, suggesting that sex steroids may organize adult responses to hantavirus earlier during ontogeny.

Hantaviruses are negative-sense RNA viruses (family *Bunyaviridae*) encompassing over 20 different viruses that are each carried by a different host species, with rodents serving as the primary reservoirs (18). Field surveys of several rodent species, including brush mice, deer mice, harvest mice, bank voles, and cotton rats, indicate that males are more commonly infected than females (4, 8, 11, 19, 20, 27). Because these studies used serology to determine hantavirus infection, sex differences in infection could reflect either a lack of infection or the absence of sustained antibody production in females. Experimental inoculation of female rodents with hantavirus, however, illustrates that females produce long-lasting, detectable antibody (22). Alternatively, sex differences in hantavirus prevalence may reflect differences in endocrine-immune interactions (15). The extent to which sex steroids affect immune responses against hantavirus infection has not been examined.

In contrast to other rodent species, sex differences in hantavirus prevalence have not been reported consistently among natural populations of Norway rats. Among adult rats, however, males (90%) tend to be infected with Seoul virus more often than females (75%) (7, 10). Seoul virus is hypothesized to be transmitted via wounding, and adult male rats are more likely to be wounded than either females or juvenile males (10). Thus, sex differences in hantavirus prevalence may reflect complex interactions between behavior and physiology. The first goal of this study was to control for sex differences in exposure and determine whether males were more susceptible to hantavirus infection than females. At 70 to 80 days of age, 5 to 10 male and 5 to 10 female Long Evans rats (*Rattus norvegicus*) were inoculated with either 10^{-4}, 10^{-3}, 10^{-2}, 10^{2}, 10^{4}, or 10^{6} PFU of Seoul virus (strain SR-11) suspended in 0.2 ml of Eagle minimum essential medium (with Earle's salts; Meditach Cellgro, Va.). Seoul virus was obtained from the U.S. Army Medical Research Institute of Infectious Diseases (Ft. Detrick, Md.), where the virus was isolated from neonatal rat brains and passaged four times in Vero E6 cells. Blood samples were obtained from each animal prior to infection and then 10, 20, 30, and 40 days postinoculation under anesthesia with methoxyflurane vapors (Metofane; Schering Plough, Union, N.J.).

Plasma was used to detect anti-Seoul virus immunoglobulin G (IgG) using an enzyme-linked immunosorbent assay in which microtiter plates were coated overnight at 4°C with gamma-irradiated Vero E6 cells infected with Seoul virus or gamma-irradiated uninfected Vero E6 cells diluted 1:500 in carbonate buffer. Thawed plasma samples, as well as positive control samples (i.e., pooled plasma from rats previously determined to have anti-Seoul virus IgG) and negative control samples (i.e., pooled plasma from Seoul virus-naive rats), were diluted 1:100 in phosphate-buffered saline (PBS)–Tween (PBS-T) with 2% fetal bovine serum and added in duplicate to antigen-coated wells containing either infected or uninfected Vero E6 cells. The plates were sealed, incubated at 37°C for 1 h, and washed with PBS-T, and secondary antibody (Kirkegaard and Perry Laboratories, Gaithersburg, Md.; alkaline phosphatase-conjugated anti-rat IgG [heavy plus light chains], horseradish peroxidase-conjugated anti-rat IgG1, or horseradish peroxidase-conjugated anti-rat IgG2a diluted 1:400 in PBS with 2% fetal bovine serum) was added. The plates were resealed, incubated for 1 h at 37°C, and washed with PBS-T, and substrate buffer (0.5 mg of *p*-nitrophenyl phosphate per ml diluted in diethanolamine substrate buffer for alkaline phosphatase reactions or tetramethylbenzidine for horseradish peroxidase reactions) was added to each well. Plates were protected from light during the enzyme-substrate reaction, which was terminated after 30 to 45 min by adding 1.5 M NaOH to each well for alkaline phosphatase reactions or 2 N H_2SO_4 to each well for horseradish peroxidase reactions. The optical density (OD) was measured at 405 nm for alkaline phosphatase reactions and 450 nm for horseradish peroxidase reactions, and the average OD for each set of uninfected Vero E6 duplicates was subtracted from the average OD for each set of infected Vero E6 duplicates. Samples were considered positive if the average adjusted OD was $\geq$0.100. To minimize intra- and interplate variability, the average adjusted OD for each sample

* Corresponding author. Mailing address: Department of Molecular Microbiology and Immunology, Johns Hopkins School of Hygiene and Public Health, 615 N. Wolfe St., Baltimore, MD 21205-2179. Phone: (410) 955-3708. Fax: (410) 955-0105. E-mail: ggurrigl@jhsph.edu.

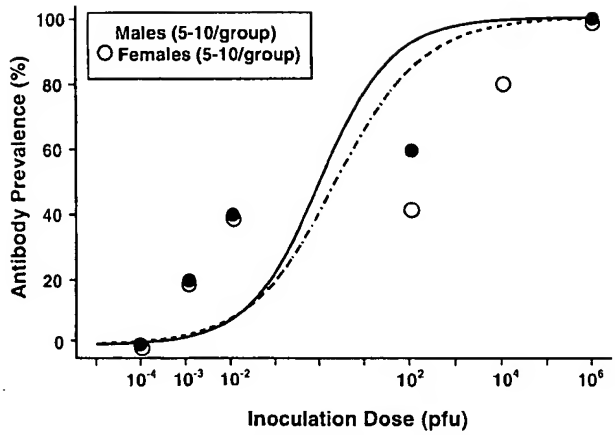

FIG. 1. Antibody prevalence among intact male and female rats inoculated with either 10^{-4}, 10^{-3}, 10^{-2}, 10^{2}, 10^{4}, or 10^{6} PFU of Seoul virus. Data are presented as percentages of individuals producing detectable antibody (i.e., adjusted average OD $\geq$ 0.100) against Seoul virus by day 40 postinoculation, with the fitted logistic regression curves for both males (solid line) and females (dashed line) included. Equal percentages of males and females seroconverted in response to each dose of Seoul virus ($P > 0.05$ in each case).

was expressed as a percentage of its plate-positive control OD for statistical analyses (9).

Antibody prevalence (i.e., the number of animals with detectable anti-Seoul virus IgG) by day 40 postinoculation was compared between males and females using chi-square analyses. Antibody prevalence was assessed 40 days after inoculation because previous studies illustrate that hantavirus-specific antibody is detectable 15 to 30 days postinoculation (7, 14, 22). Antibody prevalence did not differ between males and females at any of the six doses of Seoul virus ($P > 0.05$). Logistic regression was used to compare the infective-dose (ID) curves and estimate the 50% ID (ID_{50}). The ID_{50} did not differ significantly between males (mean $\pm$ standard deviation, 1.1 $\pm$ 2.0 PFU) and females (7.6 $\pm$ 2.0 PFU) (Fig. 1).

Although the prevalence of males and females that became infected did not differ, studies of other viral infections suggest that patterns of immune responses differ between the sexes and are mediated by sex steroid hormones (1, 15, 29). Thus, males and females may differ because testosterone suppresses and estradiol enhances several aspects of immune function (1, 15, 17, 24, 26, 29). The second aim of this study was to examine whether adult sex steroid hormone concentrations influence immune responses and virus shedding following hantavirus infection. Immunologically, patterns of helper T (Th) cell responses (i.e., Th1 or Th2) differ between males and females, with males exhibiting elevated Th1 responses (i.e., elevated gamma interferon, interleukin-2 [IL-2], and IgG2a levels) and females exhibiting increased Th2 responses (i.e., higher IL-4, IL-5, IL-6, and IL-10 levels) (5, 12, 13). Treatment of males with estradiol and females with testosterone prior to infection with pathogens, such as coxsackievirus, reverses the Th responses, suggesting that hormones can modify immune responses to virus infection (12, 13). To determine whether adult sex steroid hormone concentrations influence immune responses and virus shedding following hantavirus infection, at 70 to 80 days of age 20 male and 20 female rats were bilaterally gonadectomized under ketamine (80 mg/kg of body mass)–xylazine (6 mg/kg) anesthesia (Phoenix Pharmaceutical, St. Joseph, Mo.) and given 2 weeks to recover from surgery. After recovery, 10 castrated males were each subcutaneously implanted with a 30-mm Silastic capsule (inside diameter [i.d.] = 1.47 mm, outside diameter [o.d.] = 1.96 mm) containing 20 mm of testosterone propionate (Sigma, St. Louis, Mo.). The remaining 10 castrated males, as well as 10 intact males, were each implanted with an empty capsule of equal length. Ten ovariectomized females were each subcutaneously implanted with a 15-mm Silastic capsule (i.d. = 1.47 mm, o.d. = 1.96 mm) containing 10 mm of estradiol benzoate (Sigma). The remaining 10 ovariectomized females and 9 intact females were each implanted with an empty Silastic capsule of equal length. Silastic capsule length was based on previous reports that these hormone doses (i.e., the length of the Silastic capsule) are sufficient to maintain physiological testosterone and estradiol concentrations in male and female rats, respectively (25). At the time the Silastic capsules were implanted, all animals received an intraperitoneal inoculation of 10^4 PFU of Seoul virus (strain SR-11) suspended in 0.2 ml of Eagle minimum essential medium (i.e., the ID_{90} from the first experiment). Blood, saliva, and fecal samples were then obtained from each animal on days 0, 10, 15, 20, 30, and 40 postinoculation under anesthesia with methoxyflurane vapors. Saliva samples were collected from anesthetized rats after injecting them intraperitoneally with 2.5 mg of pilocarpine HCl (Sigma) per kg of body mass suspended in 0.9% sterile saline (6). After samples were collected on day 40 postinoculation, animals were killed and seminal vesicles were removed from the males and weighed as an index of long-term testosterone concentrations. All procedures described in this paper were approved by the Johns Hopkins Animal Care and Use Committee (protocol number RA98H536) and the Johns Hopkins Office of Health, Safety, and Environment (registration number A9902030102).

Relative seminal vesicle weights (i.e., corrected for body mass) were higher among intact males (0.282 $\pm$ 0.13 g) and castrated males treated with testosterone (0.326 $\pm$ 0.12 g) than among castrated males (0.095 $\pm$ 0.06 g) [$F(2, 29) = 12.75$, $P < 0.05$]. Plasma testosterone concentrations in males and estradiol concentrations in females were assayed by radioimmunoassay using the manufacturer's protocols (ICN Biochemicals, Inc., Carson, Calif.). Testosterone concentrations were higher for intact males and castrated males treated with testosterone than for castrated male rats; castrated males treated with testosterone also had higher testosterone concentrations than intact males on days 10, 15, 20, and 30, but not on day 40, postinoculation [$F(10, 179) = 19.30$, $P < 0.05$] (Table 1). Plasma estradiol concentrations were higher for intact females and ovariectomized females treated with estradiol than for ovariectomized females 10, 15, 20, 30, and 40 days postinoculation; ovariectomized females treated with estradiol also had higher estradiol concentrations than intact females on days 10, 15, 20, 30, and 40 postinoculation [$F(10, 173) = 10.29$, $P < 0.05$] (Table 1).

Manipulation of testosterone concentrations in males and estradiol concentrations in females did not affect production of antibody against Seoul virus ($P > 0.05$). Overall, males had higher anti-Seoul virus IgG responses than females on days 20, 30, and 40 postinoculation, regardless of hormone treatment [$F(5, 353) = 18.72$, $P < 0.05$] (Table 2). Male rats also had higher anti-Seoul virus IgG2a responses than females on days 30 and 40 postinoculation despite hormone manipulation [$F(5, 353) = 7.81$, $P < 0.05$] (Fig. 2A). In contrast, females tended to show higher IgG1 responses than males on days 30 and 40 postinoculation, though this did not reach statistical significance ($P > 0.05$) (Fig. 2B).

Viral RNA was identified using nested reverse transcription-PCR (RT-PCR), and the presence of virus in saliva and feces was used to determine whether virus was shed. Viral RNA was isolated using a guanidine isothiocyanate procedure (3). For

TABLE 1. Sex steroid hormone concentrations[a]

Hormone and group	Hormone concn (mean ± SE) on day postinoculation[b]					
	0	10	15	20	30	40
Testosterone						
Intact males	0.69 ± 0.17*	0.84 ± 0.17*	1.13 ± 0.36*	0.92 ± 0.25*	0.77 ± 0.19*	0.70 ± 0.13*
Castrated males	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
T-treated males	0.00 ± 0.00	8.24 ± 0.74*†	6.28 ± 0.91*†	6.62 ± 1.18*†	2.73 ± 0.42*†	0.71 ± 0.28*
Estradiol						
Intact females	25.8 ± 6.81*	27.0 ± 5.57*	20.8 ± 8.39*	25.9 ± 7.78*	38.2 ± 10.1*	55.2 ± 10.2*
Ovx females	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
E_2-treated females	0.00 ± 0.00	166.6 ± 20.6*†	123.1 ± 21.9*†	87.5 ± 8.9*†	162.3 ± 18.8*†	109.7 ± 19.3*†

[a] Sex steroid hormone concentrations in males and females that either were intact, gonadectomized (i.e., males were castrated and females were ovariectomized [Ovx]), or gonadectomized with sex steroids replaced (i.e., gonadectomized males received testosterone [T]-filled capsules and gonadectomized females received estradiol [E_2]-filled capsules).

[b] Testosterone levels are in nanograms per milliliter, and estradiol levels are in picograms per milliliter. An asterisk indicates that intact and hormone-treated animals had higher hormone concentrations than their gonadectomized counterparts on the corresponding day, based on an analysis of variance ($P < 0.05$). A dagger indicates that hormone-treated animals had higher sex steroid concentrations than their intact counterparts on the corresponding day, based on an analysis of variance ($P < 0.05$).

RNA isolation from saliva, samples were collected from each rat and added to Trizol LS reagent (Life Technologies, Rockville, Md.) at a 3:1 ratio, with RNase-free glycogen (10 μg) added as a carrier. For RNA isolation from feces, approximately 100 mg of feces was homogenized in Tris-EDTA buffer (pH 8.0) and centrifuged at 12,000 × *g* for 10 min at 4°C; supernatants were collected, incubated with proteinase K (50 μg/ml; Life Technologies) and 0.5% sodium dodecyl sulfate at 50°C for 30 min to digest proteins, and then added to Trizol LS at a 3:1 ratio. To separate, precipitate, and resuspend viral RNA, the manufacturer's protocol was used (Trizol LS; Life Technologies).

For RT-PCR, a 280-bp nucleotide sequence of the SR-11 small (S) genome was amplified using two 20-bp primers, HTN-S4 (5′ GATAGGTGTCCACCAACATG 3′) and HTN-S6 (5′ AGCTCTGGATCCATGTCATC 3′), that amplified positions 979 through 1259 (3). The DNA fragment obtained from the RT-PCR was further amplified using primers HTN-S3 (5′ GCCTTCTTTTCTATACTTCAGG 3′) and HTN-S5 (5′ CCAGGCAACCATAAACATAAC 3′), designed to amplify a 176-bp nucleotide sequence (positions 1031 through 1207). First-strand cDNA was prepared using the GeneAmp RNA PCR kit protocol (Perkin-Elmer, Branchburg, N.J.), incubated in a DNA thermocycler (Techne Genius) at 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min, and then held at 4°C. The reaction mixture contained 5 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates, 1 U of RNase inhibitor, and 2.5 U of murine leukemia virus reverse transcriptase. The positive control was SR-11 RNA isolated from virus stock, and the negative control was diethyl pyrocarbonate water that was included in the cDNA syntheses and primary and secondary amplifications.

The 280-bp sequence was amplified in a 100-μl reaction mixture containing 20 μl of the cDNA, 0.3 μM HTN-S6 primer, and 2.5 U of polymerase (AmpliTaq; Perkin-Elmer). Reactions were amplified for one cycle at 94°C for 3 min and 40 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 60 s, followed by 10 min at 72°C. The nested 176-bp sequence was amplified in a 100-μl reaction mixture containing 2 μl of the product of the first DNA amplification, 20 μM HTN-S3 primer, 20 μM HTN-S5 primer, 10 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates, and 2.5 U of polymerase. Nested-PCR products were amplified using the same cycle series as was used for the primary amplification. The PCR products were electrophoresed on a 4% gel (3% NuSieve plus 1% SeaKem; FMC Bioproducts, Rockland, Maine), stained with ethidium bromide, and examined for bands of the appropriate size. Randomly selected positive PCR products from saliva and fecal samples from males and females, as well as positive and negative control products, were purified using QIAquick (Qiagen, Valencia, Calif.) and sequenced.

Virus shedding in saliva and feces was not altered by hormone manipulation ($P > 0.05$) (Table 3). Overall, more males shed virus in saliva than females 10 days ($\chi^2 = 3.82$, $df = 1$, $P = 0.051$) and 30 days ($\chi^2 = 8.19$, $df = 1$, $P < 0.05$) after inoculation with Seoul virus (Table 3). The prevalence of Seoul virus in feces also differed between males and females on day 30 postinoculation; more males shed virus in feces than females

TABLE 2. Plasma anti-Seoul virus IgG responses[a]

Group	Anti-Seoul virus IgG response (mean ± SE) on day postinoculation[b]					
	0	10	15	20	30	40
Intact males	0.8 ± 0.6	4.9 ± 3.0	84.0 ± 22.0	106.0 ± 19.0*	332.0 ± 47.0*	342.1 ± 56.0*
Castrated males	1.0 ± 0.7	1.0 ± 1.0	82.0 ± 21.0	106.0 ± 27.0*	280.0 ± 71.0*	387.3 ± 84.0*
T-treated males	1.0 ± 0.7	2.0 ± 0.9	33.0 ± 10.0	108.0 ± 14.0*	314.0 ± 41.0*	426.7 ± 43.0*
Intact females	3.0 ± 1.0	9.0 ± 4.0	36.0 ± 10.0	60.0 ± 14.0	189.0 ± 55.0	219.6 ± 63.0
Ovx females	2.0 ± 0.8	4.0 ± 2.0	7.0 ± 3.0	54.0 ± 16.0	187.0 ± 56.0	209.2 ± 53.0
E_2-treated females	3.0 ± 1.0	8.0 ± 2.0	19.0 ± 6.0	39.0 ± 8.0	178.0 ± 42.0	209.1 ± 39.0

[a] Plasma anti-Seoul virus IgG responses in males and females that either were intact, gonadectomized (i.e., males were castrated and females were ovariectomized [Ovx]), or gonadectomized with sex steroids replaced (i.e., gonadectomized males received testosterone [T]-filled capsules and gonadectomized females received estradiol [E_2]-filled capsules).

[b] Data are presented as IgG units, in which the mean OD of each test sample was divided by the OD of the positive control sample run on the same microtiter plate. An asterisk indicates that males had higher IgG responses than females, regardless of hormone manipulation, based on an analysis of variance ($P < 0.05$).

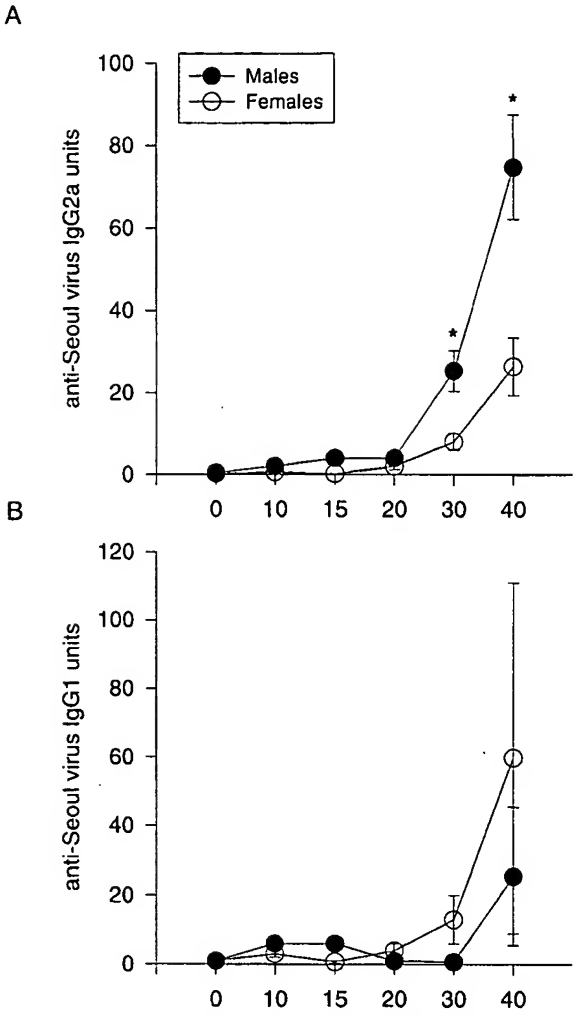

FIG. 2. (A) Plasma anti-Seoul virus IgG2a responses (mean ± standard error) in male and female rats. (B) Plasma anti-Seoul virus IgG1 responses (mean ± standard error) in male and female rats. Blood samples were collected 0, 10, 15, 20, 30, and 40 days following inoculation with Seoul virus. For calculation of IgG2a or IgG1 units, the mean OD of each test sample was divided by the OD of the positive control sample run on the same microtiter plate. Because neither gonadectomy nor hormone replacement had an effect on antibody production, responses from the different treatments groups were collapsed and graphed together. An asterisk indicates that males had higher IgG2a responses than females ($P < 0.05$).

($\chi^2 = 6.88, df = 1, P < 0.05$) (Table 3). In general, males shed virus in saliva and feces more consistently than females, regardless of hormone manipulation (Table 3). The PCR product obtained from saliva and feces of males and females was sequenced and verified as Seoul virus DNA.

Sex differences in the prevalence of hantavirus infection have been observed in several natural rodent populations, including deer mice, brush mice, harvest mice, bank voles, and cotton rats (4, 8, 11, 19, 20, 27). In each case, males are infected more often than females. Field studies of Norway rats suggest that sex differences in hantavirus prevalence reflect sex differences in behaviors, like aggression, that increase the likelihood of males being infected (10). High circulating testosterone concentrations increase the probability of engaging in aggressive encounters in several vertebrate species (21). In addition to modulating aggression, sex steroid hormones can affect immune responses against infection. Studies of viral infections, such as coxsackievirus, suggest that sex differences in both the prevalence and intensity of infection are due to differences in endocrine-immune interactions (12, 13).

Despite the known effects of sex steroids on infection, in the present study, manipulation of adult sex steroids had no effect on immune responses or virus shedding following exposure to Seoul virus. Specifically, males had higher antibody responses and shed virus longer than females, regardless of adult hormone manipulation. Sex steroid hormones affect physiology and behavior at two distinct times during ontogeny (2, 16, 23). During perinatal development, sex steroids cause sex differences in the differentiation or organization of central and peripheral structures. In adulthood, exposure to sex steroids serves to activate preexisting hormonal circuits. The data from the present study may suggest that sex steroid hormones are not involved in hantavirus infection. Alternatively, these data may illustrate that manipulation of activational sex steroids does not alter responses to infection because the hormonal circuitry was organized earlier during development. If sex steroids organize adult responses to infection, then manipulation of neonatal sex steroids should alter adult responses to hantavirus infection.

Regardless of hormone manipulation, males had higher anti-Seoul virus IgG2a responses than females. Recent data from our laboratory indicate that following Seoul virus inoculation, males have elevated IL-2 and gamma interferon concentrations and females have elevated IL-4 responses (S. L. Klein and G. E. Glass, unpublished data). Taken together, these data suggest that males may have higher Th1 responses to hantavirus infection than females. Studies of other viral infections in rodents suggest that females typically have higher Th2 re-

TABLE 3. Virus shedding[a]

Sample and group	No. of virus-shedding rats/total on day postinoculation[b]				
	10	15	20	30	40
Saliva samples					
Intact males	6/11	7/10	6/11	6/11	6/11
Castrated males	4/9	4/9	6/9	5/9	8/9
T-treated males	9/10	7/10	4/10	6/10	7/10
Total males	19/30*	18/29	16/30	17/30*	21/30
Intact females	3/9	6/9	5/9	2/9	2/9
Ovx females	4/10	7/10	2/10	2/10	6/10
E_2-treated females	3/10	10/10	3/10	1/10	6/10
Total females	10/29	23/29	11/29	5/29	14/29
Fecal samples					
Intact males	5/11	4/11	4/11	5/11	1/11
Castrated males	6/9	5/9	7/9	4/8	1/9
T-treated males	4/10	6/10	7/10	7/9	1/10
Total males	15/30	15/30	18/30	16/29*	3/30
Intact females	7/9	4/9	4/9	1/8	0/9
Ovx females	9/10	4/10	6/10	2/10	2/10
E_2-treated females	6/9	5/10	8/10	2/10	1/10
Total females	22/28	13/29	18/29	5/28	3/29

[a] Virus shedding in saliva and feces from males and females that either were intact, gonadectomized (i.e., males were castrated and females were ovariectomized [Ovx]), or gonadectomized with sex steroids replaced (i.e., gonadectomized males received testosterone [T]-filled capsules and gonadectomized females received estradiol [E_2]-filled capsules).

[b] An asterisk indicates that more males shed virus than females on the respective day postinoculation, based on chi-square analyses ($P < 0.05$).

sponses than males and that this is due, in part, to the effects of estrogens on cytokine production (12). In the present study, females tended to produce higher IgG1 responses than males. In contrast to estrogens, androgens promote differentiation of $CD4^+$ T cells to a Th1 phenotype (12). In the present study, however, castrated and intact males had similar IgG2a responses, suggesting that increased Th1 responses are not contingent on the direct effects of androgens.

High antibody responses in males may indicate that males have more efficient immune responses against infection than females. This outcome seems unlikely given the rapid increase and long duration of virus shedding in males compared to females. Alternatively, males may have higher antibody responses than females because virus replication is increased in males. Higher Th1 responses are associated with increased susceptibility to infections caused by coxsackievirus and Sindbis virus in mice (12, 28). Although quantitative analyses were not conducted, males shed Seoul virus longer than females, suggesting that higher Th1 responses among males may be a consequence of increased virus replication.

In summary, although males and females are equally susceptible to infection with Seoul virus, males shed virus longer and produce higher Th1 responses against Seoul virus than females. Increased virus shedding among males may explain why males are more likely to acquire Seoul virus infection following aggressive encounters among natural populations of Norway rats (10). In the present study, manipulation of adult sex steroid hormones did not alter immune responses or virus shedding following inoculation with Seoul virus. Although sex steroid hormones may not mediate sex differences in response to hantavirus infection, sex differences in infection among adults may be altered by sex steroids earlier during development. Alternatively, sex differences in infection may reflect other neuroendocrine changes, such as differences in glucocorticoids, that may affect responses to Seoul virus infection.

This research was supported by NASA grant NCC5-305 (G.E.G.) and NIH NRSA AI 10324 (S.L.K.).

We thank Connie Schmaljohn and Cindy Rossi for providing hantavirus reagents, Alan Scott and Aimee Marson for assistance with PCR development, and Randy Nelson and Deborah Drazen for assistance with radioimmunoassays. We also thank Diane Griffin and Alan Scott for helpful comments on early drafts of the manuscript.

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